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Photocleavage of the Polypeptide Backbone by 2-Nitrophenylalanine

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SUMMARY

Photocleavage of the polypeptide backbone is potentially a powerful and general method to activate or deactivate functional peptides and proteins with high spatial and temporal resolution. Here we show that 2-nitrophenylalanine is able to photochemically cleave the polypeptide backbone by an unusual cinnoline-forming reaction. This unnatural amino acid was genetically encoded in *E. coli*, and protein containing 2-nitrophenylalanine was expressed and site-specifically photocleaved.

INTRODUCTION

Natural proteolytic activities are involved in a wide array of biological processes including zymogen activation, protein degradation, and the removal of signal peptides. The photochemical regulation of such processes is increasingly being used to probe cellular events (Pelliccioli and Wirz, 2002; Young and Deiters, 2007). The ability to photochemically initiate protein cleavage would allow spatial and temporal control over the activity, lifetime, and localization of proteins both in vitro and in living cells. Indeed, it has been shown that the protein backbone can be photocleaved selectively if the unnatural amino acid 2-nitrophenylglycine (2-NPG) is incorporated into the protein (Endo et al., 2004; England et al., 1997). However, the production of proteins containing 2-NPG by current methods is problematic—biosynthesis using chemically aminoacylated suppressor tRNAs produces small, stoichiometric amounts of protein, and semisynthetic methods are generally restricted to substitutions at C- or N-terminal sites (Bosques and Imperiali, 2003; Pellois et al., 2004; Pellois and Muir, 2005). In order to further expand our ability to control proteolysis in vitro or directly in living cells we have genetically encoded the phenylalanine derivative, 2-nitrophenylalanine (2-NPA), in *Escherichia coli* in response to the amber codon, TAG. Upon irradiation at 365 nm, the 2-NPA residue photocleaves the protein backbone specifically at the site of incorporation.

RESULTS

Photocleavage of a Model Peptide

We anticipated that incorporation of 2-NPA into proteins might allow photocleavage of the polypeptide backbone based on the photochemistry of (2-nitrophenyl)ethane derivatives (Bühler et al., 2004; Hasan et al., 1997; Walbert et al., 2001), which like the more common 2-nitrobenzyl group (Bley et al., 2008; Momotake et al., 2006) are used as photochemical caging groups. Specifically, alkoxy substituents β to the nitrophenyl group undergo photochemical elimination reactions, suggesting that an amide group might behave similarly. To test this notion, model peptide **1** containing the 2-NPA group was synthesized (Figure 1). When peptide **1** (10 μM) was photolysed at 365 nm in phosphate-buffered saline at pH 7.4 (PBS), photocleavage at the expected site was observed in greater than 95% yield. The quantum yield Φ of the photocleavage reaction was determined by chemical actinometry using 2-nitrobenzaldeyhyde as a known standard (see Supplemental Data available online). The quantum yield at 365 nm was determined to be 0.07 ± 0.01. Analysis of the products of the photocleavage reaction by mass spectrometry, and by ^{1}H and ^{13}C 1D and 2D nuclear magnetic resonance (NMR) revealed two peptide fragments: a C-terminal carboxylate group, and an N-terminal cinnoline group (rather than the expected C-terminal amide and N-terminal substituted olefin). Based on the structure of the products and previous studies of 2-nitrophenylethane photolysis, we propose the putative mechanism outlined in Figure 2. Upon photolysis, the nitrobenzyl group rearranges to the α-hydroxy-substituted nitrosophenyl group as expected. The nitroso group then undergoes an addition reaction with the N-terminal amide group (Ayyangar et al., 1989) to generate the cyclic azo product **6**. Subsequent hydrolysis of the activated carbonyl group affords the terminal cinnoline and carboxylate products. Support for this mechanism was obtained by photolysis of **1** in a solution of 30% methanol in PBS. Under these conditions, the methyl ester was formed in addition to the carboxylic acid hydrolysis product **2** (Figure S1). Consistent with this mechanism, photolysis of a similar peptide where the amino terminus was unprotected gave several side products, which are likely the result of intramolecular cyclization reactions (Figures S2A–S2E).

Genetic Incorporation of 2-NPA into Proteins in *E. Coli*

Given that photolysis of 2-NPA efficiently cleaves the polypeptide backbone, we next genetically encoded this unnatural amino acid in *E. coli* according to published procedures (Wang et al., 2001). Briefly, an amber suppressor *Methanococcus jannaschii* $tRNA^{Tyr}_{CUA}$ and tyrosyl-tRNA synthetase pair, which is orthogonal in *E. coli*, was evolved to accept 2-NPA as a substrate instead of its natural substrate, tyrosine. A library of 6×10^9 active site mutants (in which residues Tyr32, Leu65,

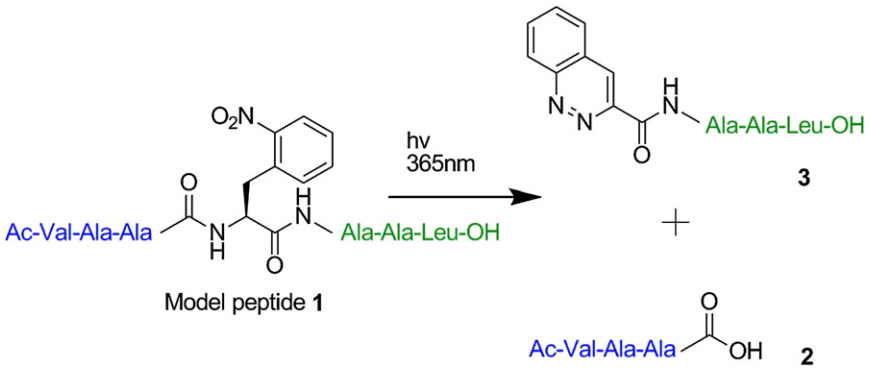

Figure 1. Photolysis of 1 Leads to Cleavage of the Peptide Backbone and Generates a C-Terminal Carboxylate Group and N-Terminal Cinnoline Group

Ala67, His70, Phe108, Gln109, Tyr114, Asp158, Ile159, and Leu162 were randomized with NNK [N = any A, T, G, or C; K = T or G]) was subjected to sequential rounds of positive and negative selection. Positive selection is based on the ability of aminoacyl-tRNA synthetases specific for 2-NPA to suppress an amber codon in the chloramphenicol acetyl transferase gene in the presence of 2-NPA and chloramphenicol. The negative selection is based on expression of a toxic barnase gene containing two amber stop codons in the absence of 2-NPA. Aminoacyl-tRNA synthetases that aminoacylate the amber suppressor tRNA with endogenous amino acids generate active barnase protein (BARN), leading to cell death. After three rounds of selection, an aminoacyl-tRNA synthetase was isolated that incorporates 2-NPA with > 95% fidelity based on SDS denaturing polyacrylamide gel analysis of the expression of an Asp61TAG mutant of bacteriophage T4 lysozyme (T4L) containing a C-terminal His6 tag in the presence and absence of 2-NPA (Figure S3A). ESI-TOF mass spectrometry confirmed incorporation of 2-NPA (Figure S3B). The overall yield was 2.1 mg/l after Ni-NTA and fast protein liquid chromatography (FPLC) purification when cells were grown in minimal glycerol minimal media with leucine (GMML) media in the presence of 1 mM 2-NPA (the yield of wild-type protein was 9.6 mg/l). The enzyme has ten mutations in its active site; Y32G, L65H A67G, H70G, F108L, Q109S, Y114S, D158T, I159Y, and L162D. Six other aminoacyl-tRNA synthetases with a high degree of homology were also identified, but all displayed lower fidelity for 2-NPA incorporation (Table S1).

Expression and Photocleavage of a Model Protein, T4 Lysozyme

To determine whether incorporation of 2-NPA results in photocleavage of a folded protein, Ala82 in T4 lysozyme was substituted with 2-NPA. After Ni-NTA and subsequent FPLC purification, the protein was photolysed with > 300 nm light in PBS buffer for varying periods and the products were then analyzed by SDS-PAGE (Figure 3). Two fragments corresponding to the photocleaved amino- and carboxy-terminal fragments of the parent protein migrated on SDS-PAGE at the expected molecular masses. A yield of 22% ± 5% cleavage was obtained after irradiation for 60 min as determined by optical densitometry of a Coomassie-stained gel and confirmed by mass spectral analysis. The two fragments were unambiguously identified as photocleavage products of the parent protein by trypsin digestion and nano-LC tandem mass spectrometry (Figures S4A and B). Fragments containing the histidine tag were also identified by western blotting using antihistidine tag antibodies. The photocleavage reaction of the folded protein did not afford

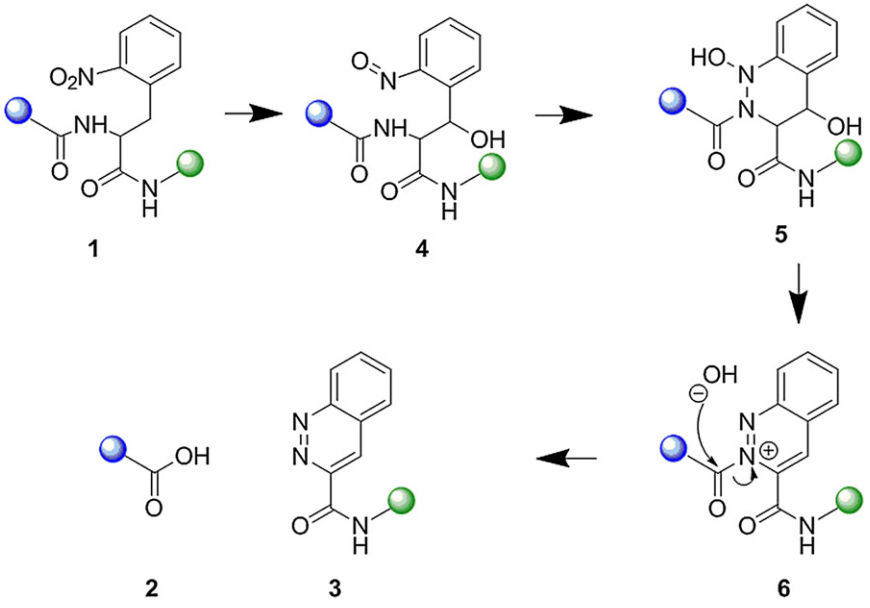

Figure 2. Proposed Mechanism of Photocleavage Reaction

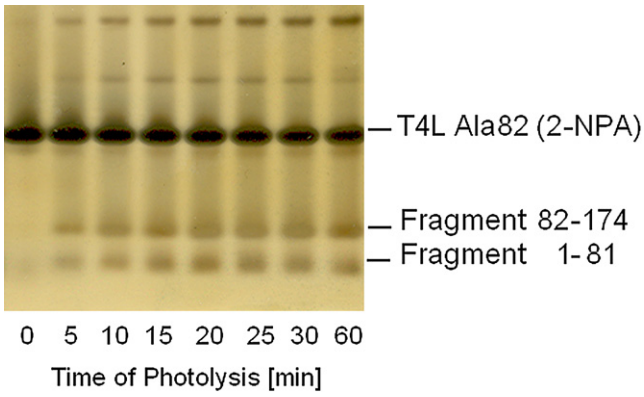

Figure 3. Photolysis of Ala82 (2-NPA) T4L in PBS Solution Using a 300 nm Cut-Off Filter

Aliquots were taken at several time points, separated by SDS-PAGE, and visualized by silver staining.

near-quantitative cleavage as was seen with the model peptide. The most likely source of side products is intramolecular reactions with side-chain amines or amides, as was observed upon photolysis of the unprotected model peptide.

Photocleavage by Incorporation of 2-NPA at Various Residues of T4 Lysozyme

To determine if the position of the amino acid in the protein significantly affects the efficiency of photocleavage, we expressed several variants of T4L in which 2-NPA was incorporated at residues Lys60, Asp61, Lys65, Lys68, Asp72, Ala73, and Arg80. Photolysis of the purified proteins showed that all the proteins were cleaved, and that the placement of 2-NPA does somewhat affect the yield of photocleavage and quantity of side products produced (Figure 4), although there was no obvious correlation with local secondary structure or sequence. The Asp61TAG mutant afforded the most cleavage product with the least side product formation. The maximal yield of cleavage (30% ± 5%) was observed after 25 min of photolysis as determined by optical densitometry.

DISCUSSION

The 2-NPA group is able to photochemically cleave the polypeptide backbone when chemically or biosynthetically incorporated into a protein. By analyzing the products of the photolysis of a model peptide, we identified an unusual photocleavage reaction which generates a C-terminal carboxylate group and N-terminal cinnoline ring. Thus the mechanism of peptide bond cleavage is distinct from that of 2-NPG. The ability to genetically encode 2-NPA expands the range of in vitro and in vivo experiments to which the photocleavable amino acid can be applied. The yield of photocleavage is less than 50%, likely due to intramolecular cyclization products of reactive intermediates. Thus the current methodology will likely be useful for generating active proteins from photocaged precursors (e.g., zymogen activation, release of a biologically active peptides), but will be less useful for the inactivation of proteins in cells (unless the side products are also inactive). The synthetase that we have developed to accept 2-NPA should also be useful as a template to genetically encode amino acids with enhanced photochemical properties such as the dimethoxy substituted analog, which has a higher quantum yield and longer wavelength excitation. Attempts to genetically encode 2-NPG in *E. coli* have not yet been successful despite several efforts with distinct orthogonal tRNA/aminoacyl-tRNA synthetase pairs. In addition, the 2-NPA is a close analog of phenylalanine and would be expected to minimally perturb protein structure, dynamics, and function, in contrast to the β-branched 2-NPG residue, which might be expected to affect the conformation of the polypeptide backbone.

SIGNIFICANCE

We have demonstrated that 2-nitrophynlalanine, when incorporated chemically or biosynthetically into a polypeptide, is able to site-specifically cleave the polypeptide backbone upon irradiation with 365 nm light. In a model peptide, the photocleavage reaction yields a C-terminal carboxylate group and N-terminal cinnoline group as the major cleavage products in excellent overall yields. A maximum cleavage efficiency of ~30% was achieved when the 2-NPA group was biosynthetically incorporated into proteins, which is likely due to competing intramolecular cyclization reactions with nearby nucleophilic groups. Because 2-NPA is a close analog of phenylalanine, its incorporation into peptides or proteins is expected to minimally perturb their structure and function. Thus, the 2-NPA can be used to activate or inactivate biologically active peptides with high temporal

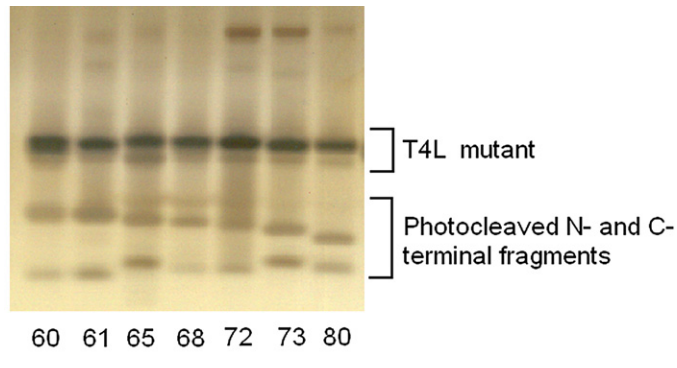

Figure 4. Photolysis of Various T4L Mutant Proteins Containing 2-NPA at the Indicated Sites

Polypeptides of the expected sizes are observed.

and spatial resolution, both in vitro and in living cells (Lemke et al., 2007). In the case of proteins (where the photocleavage efficiency will typically be lower), this methodology will be most useful for generating biologically active species from inactive precursors, rather than directly inactivating proteins where a significant change in activity will be more difficult to quantify.

EXPERIMENTAL PROCEDURES

General

All chemicals were obtained from commercial sources and used without further purification. NMR data were collected on a Bruker DRX-600 instrument with chemical shifts recorded relative to residual solvent peaks of (CD3)2SO (1H 2.50 ppm, 13C 39.52 ppm). Mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, CA) and at the Genomics Institute of the Novartis Research Foundation (La Jolla, CA). Standard molecular biology techniques (Sambrook et al., 2001) were used throughout. DH10b *E. coli* was used for routine cloning, DNA propagation, library selections, and protein expression.

Peptide Photocleavage

2-Nitrophenylalanine as the FMOC protected derivative was obtained from CSPS Pharmaceuticals, Inc. (San Diego, CA). The model peptide 1 was synthesized by Anaspec (San Jose, CS) and purified by high-performance liquid chromatography (HPLC) with a C18 column (SUPELCO, Discovery, 250 mm × 4.6 mm, 5 μm) using a water: 5%–95% acetonitrile gradient over 60 min with both solvents containing 0.05% formic acid. A solution of peptide 1 (10 μM) in PBS (pH 7.4) was photolyzed using an Oriel 500W Mercury arc lamp fitted with a water filled/water cooled liquid filter. The beam was filtered through a 365 nm band-pass filter (FWHM 10 nm, Asahi Spectra USA Inc.) and irradiated in a quartz cuvette with a 5.0 mm path length at room temperature. In order to collect enough material for NMR characterization, 10 mg was photolysed for 40 min in a large Pyrex dish atop a DNA illumination table (Fotodyne, Fotoprep) fitted with a 302 nm light source. The solution was concentrated by rotary evaporation and the products separated by HPLC as described above for the purification of the intact peptide.

Quantum Yield of Peptide Photolysis

The quantum yield was determined by chemical actinometry using 2-nitrobenzylaldehyde as a standard. (Kuhn, 2004). Actinometry was performed in low optical density mode due to the poor solubility of the peptide in an aqueous PBS. Both peptide and 2-nitrobenzaldehyde solutions were 10 μM in PBS. An Oriel 500 W Mercury arc lamp fitted with a water filled/water cooled liquid filter was used as a light source, and the beam was filtered through a 365 nm band-pass filter (FWHM 10 nm, Asahi Spectra USA Inc.) and irradiated in a quartz cuvette with a 5.0 mm path length. The time-course for photoreaction of the peptide and 2-nitrobenzaldehyde was followed by HPLC analysis using a Waters 2690 separations module equipped with a C18 column (SUPELCO, Discovery, 250 mm × 4.6 mm, 5 μm) and a Waters 996 photodiode array detector. Pump A delivered water/0.1% formic acid, and pump B delivered acetonitrile/0.1% formic acid. The components were separated by a linear gradient over 25 min from 1% to 90% buffer B. Each sample was analyzed five times.

Genetic Selection of a Mutant Aminoacyl-tRNA Synthetase Specific for 2-NPA

A library of *M. jannaschii* TyrRS synthetase mutants was constructed as previously described (Xie and Schultz, 2005) where residues Tyr32, Leu65, Ala67, His70, Phe108, Gln109, Tyr114, Asp158, Ile159, and Leu162 were randomized (NNK). After three positive and three negative rounds of selection (Xie and Schultz, 2005), aminoacyl-tRNA synthetases with the sequences listed in Table S1 were enriched. Clone 1 has the highest fidelity for incorporation of 2-NPA.

Bacteriophage T4 Lysozyme Mutagenesis

A C-terminal, hexahistidine-tagged, bacteriophage T4 lysozyme gene (containing N2D, C54T, C97A mutations) was amplified from pLeiT4L-wt (Brustad et al., 2008) and inserted into the vector pBAD/JYAMB (Mehl et al., 2003) using the NcoI and KpnI restriction sites, thereby exchanging the myoglobin gene for that of T4 lysozyme and creating pBAD-T4L-wt. Standard site directed mutagenesis was used to create several mutants of the T4L gene (Stratagene, Quikchange II). The primers used for mutagenesis are given in Supplemental Data. All plasmid constructs were verified by sequencing.

Protein Expression and Purification

Bacteriophage T4 lysozyme containing 2-NPA at position 61 was expressed in *E. coli* DH10B cells harboring the two plasmids pBAD-T4L-61TAG and pBK-TyrRS-2NPA (clone 1). A 1 l GMML culture containing 50 μg/ml kanamycin, 50 μg/ml ampicillin, and 2-NPA (1 mM) was grown at 37°C to an OD600 of 0.5. The culture was then induced by adding arabinose to a final concentration of 0.05% and incubated with shaking at 30°C for 18 hr. Cells were collected by centrifugation, and the His-tagged T4 lysozyme was purified by Ni-NTA affinity chromatography under native conditions according to the manufacturer's protocol (QIAexpressionist, QIAGEN). Imidazole was removed by several rounds of concentration (Amicon Ultra-15, Millipore) and dilution in MES buffer A (MES buffer A: 50 mM MES, 20 mM NaCl, 1 mM EDTA [pH 6.3]; MES buffer B: same as buffer A, except containing 500 mM NaCl). The protein was then purified by FPLC using a MonoS column (Amersham Pharmacia Biotech) with a gradient of 0% to 95% MES buffer B in MES buffer A over 35 min. The protein was then exhaustively dialyzed against PBS (3000 MWCO Slide-A-Lyzer Dialysis Cassette, Pierce) to give a final yield of 2.1 mg/l.

Determination of Protein Photocleavage Products

A total of 12.5 μg protein T4L containing 2-NPA at position 82 was photolysed (>300 nm) in 1 ml PBS at room temperature. Photolysis products were separated by SDS-PAGE (4%–12%, Bis-Tris, Invitrogen) and stained (GelCode Blue, Pierce). Protein bands were excised and analyzed by LC/MS/MS after in-gel trypsin digestion, and identification of protein photo-cleavage fragments was based on the observation of tryptic peptides originating mostly from the N- and C-terminal regions of the protein, with respect to the photo-cleavage cleavage site.

Protein Photocleavage Quantitation

Optical densitometry was used to determine the extent of protein photocleavage. Briefly, a standard curve of optical density versus the amount of protein per band (as determined by the Bradford method [BCA Protein Assay Kit, Pierce]) was established. The protein samples were subjected to SDS-PAGE (4%–12% Bis-Tris, Invitrogen), stained for 4 hr (GelCode Blue, Pierce), and destained for 2 hr with several washings with milliQ water. The optical density of each band was measured (ChemiDOC XRS, QuantityOne software). In order to quantify photocleavage, several 12.5 μg samples of T4L containing 2-NPA at position 61 were photolyzed (>300 nm) in 1 ml 10-fold diluted PBS for varying periods (0, 5, 10, 15, 30, and 60 min). The samples were concentrated to 25 μl, analyzed on SDS-PAGE, and the optical density of the protein bands was measured in the same fashion as the standard curve samples.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, four figures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00038-6.

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